A comparative analysis reveals differences in the innate lymphoid cell population between adult peripheral and umbilical cord blood

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The immune system continuously develops through mammalian life. As such, the neonatal and adult immune systems differ greatly, which is reflected in the composition of leukocyte populations in umbilical cord blood (UCB). To shed light on human ILC development, we compared the ILC populations in UCB and adult peripheral blood (AB). We report here, that UCB is relatively enriched in ILC2s, while less ILC1s can be found in comparison with AB. Also, while a positive correlation was found between CD62L and α4β7 in UCB, the opposite was true in AB and a population of cells expressing both markers was found to be enriched in UCB. Considering α4β7 is a marker for murine ILC precursors, it is tempting to hypothesize that these CD62L⁺ α4β7⁺ cells are a population of human ILC precursors.
INTRODUCTION

Commensal microbiota are crucial for the development of a properly functioning immune system in all mammals. Studies using germ-free mice have shown that the colonization of the intestines and the skin at birth is an important event and greatly influences the ability of the immune system to cope with pathogenic challenge later in life\(^1,2\). Importantly though, in the past, differences have been shown between human and mouse immune development. The most pronounced being colonization of lymphoid tissues by adaptive lymphocytes which happens in mice during the first weeks after birth, while in humans lymphoid tissues are colonized before birth\(^3\). Although neonates are not completely devoid of microbes despite the seemingly sterile environment of the uterus\(^4\), umbilical cord blood (UCB) which is drawn directly at birth is a rich source of human immune cells which have had no exposure to a fully matured commensal flora. Indeed, the composition of UCB has been shown to be different as compared to adult peripheral blood (AB) and innate cells derived from UCB have been shown to respond in a more tolerogenic fashion following challenge with TLR-ligands\(^5\).

Innate lymphoid cells (ILCs) constitute a family of cells of lymphoid origin, which lack rearranged antigen receptors. ILCs are potent effector cells, secreting large amounts of cytokines once stimulated. Based on the cytokines they secrete, 3 subsets of ILCs have been described, in parallel to the known Th cell subsets: IFN\(\gamma\) producing ILC1s, interleukin (IL)-5 and IL-13 producing ILC2s and IL-17A and IL-22 producing ILC3s\(^6\). In mice, it has been described that some intestinal ILC3 subsets depend on microbiota for proper development and function, although ILC1s and ILC2s seem to develop normally in germ-free mice\(^7\)-\(^11\). However, a recent report shows that the transcriptional profile of all ILC subsets in the intestine of germ-free mice is different than conventionally housed controls\(^12\). While known for their role in maintaining homeostasis and participating in inflammatory responses in mucosal and epithelial barriers, ILCs are also present in secondary lymphoid organs (SLOs), AB, as well as in UCB\(^13\). As described above, UCB leukocytes show a relatively tolerogenic phenotype in comparison with AB and microbial colonization influences the maturation of some ILC subsets in mice. Based on the latter, it could be assumed that ILCs in UCB are less mature and potentially enriched for ILC-precursors. Therefore, we aimed here to further characterize UCB ILCs based on subdivision into the different ILC subsets (i.e. ILC1s, 2s and 3s) and expression of the membrane markers CD62L, CD45RA, CD161 and \(\alpha\beta7\), all of which are known to correlate with activation and / or maturation status of ILCs.
RESULTS AND DISCUSSION

ILC subsets are differently represented in CB as compared to AB

We analyzed mononuclear cells (MNCs) from both UCB and AB for the presence of the known ILC subsets. ILCs were gated as lymphoid cells which lack known lineage markers (CD3, CD11c, CD14, CD19, CD34 and CD94) yet express the alpha chain of the IL-7 receptor (IL-7Rα, CD127) (Fig 1A). Further gating on the different ILC subsets was done using the gating strategy reported for ILCs in human lymphoid organs. Thus, by additional staining for cKit and the chemokine receptor for Th2 cells (CRTH2), ILC1s are described as cKit CRTH2-, ILC2s as CRTH2+ and ILC3s as cKit CRTH2- (Fig. 1A). Most notably, the percentage of ILCs within the lymphocyte gate was higher in UCB

Figure 1: Composition of the ILC population in UCB and AB. (A) Gating of ILCs in AB and UCB. A gate for lymphocytes was set based on FSC and SSC profile. Subsequently, doublets (not shown) and dead cells were gated out, and a gate was set on lineage (Lin) negative cells expressing CD127 cells. Finally, ILC1s were gated as cKit CRTH2-, ILC2s as CRTH2+ and ILC3 as cKit CRTH2-. Shown are plots of a representative sample. The lineage cocktail includes monoclonal antibodies for: CD3, CD11c, CD14, CD19, CD34 and CD94. (B) Percentages of the ILC (Lin CD127+) population as part of the total live lymphocyte population in AB (n = 6) and UCB (n = 11). (C) Composition of the Lin CD127+ population in AB (black bars) and UCB (grey bars). Shown are the mean percentages of ILC1s, ILC2s and ILC3s as part of the total Lin CD127+ (ILC) population. (B, C) **: p < 0.01, ***: p < 0.001, Unpaired Student’s T-test.
Comparison of UCB and AB ILCs

(Fig. 1B) and UCB contained relatively more CRTH2⁺ ILC2s and less ILC1s than AB (Fig. 1C). These observations showed that the composition of the ILC population in UCB is different from the one in AB and may be a reflection of differences in ILC differentiation from common lymphoid precursors. The more pronounced presence of ILC2s in UCB is in agreement with a recent report. A higher frequency of ILC2s is in line with the general notion that leukocytes derived from UCB present a more tolerogenic, anti-inflammatory phenotype. In general, lack of a fully developed microbiota is accompanied by increased type II immunity in mice. In humans, antibiotics treatment in infancy correlates with increased risk for developing allergies, while children raised in farms are relatively protected. In that respect, enhanced presence of ILC2s in UCB is not surprising. Importantly, the relative increase in ILC2s we observed comes at the cost of ILC1s rather than ILC3s, while in germ free mice both intestinal ILC1s and ILC2s develop normally and defects were seen in the ILC3 populations. The defects observed in germ free mice are mostly in the NKp46 expressing ILC3 population, which are present in the intestine rather than in the peripheral blood and secondary lymphoid organs. As we examined ILCs in circulation rather than in epithelial tissues, such an effect might have been missed. Finally, examination of UCB ILC functions (e.g. cytokine production), which may very well be different between AB and UCB, will shed further light on the differential capacity of the different ILC subsets in UCB, potentially as a result of the relatively sparse encounter of these cells with microbiota.

Differences in expression of membrane markers between UCB and AB ILCs

To further address the composition of the ILC population in UCB in comparison with AB, we analyzed the expression of the cell surface proteins CD161, CD45RA, CD62L and the integrin dimer α4β7, expression of which correlates with different stages of development and activation of ILCs in both mice and humans. CD161 is an activating receptor on immature natural killer (NK) cells and functionally it is necessary for homing to mucosa and mucosal-associated lymphoid tissue (MALT) where its ligand MAdCAM-1 is expressed on endothelial cells. CD62L is a homing marker for peripheral SLOs and expressed on ILC developmental intermediates in mice. Together with CD45RA it marks a naïve-like
Figure 2: Expression of CD161, CD62L, CD45RA and α4β7 on AB and UCB ILCs. (A-D) Mean percentage (left) and MFI (middle) of CD161 (A), CD62L (B), CD45RA (C) and α4β7 (D) in total ILC population, ILC1s, ILC2s and ILC3s in AB (black bars) and UCB (grey bars). On the right, representative histograms of the expression of the respective markers in the total ILC population in AB (open histograms) and UCB (tinted histograms). (A-D) *: p < 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001, Unpaired Student’s T-test. (E) Expression of CD62L, CD45RA and α4β7 by Lin CD127-CD161+ or Lin CD127-CD161- cells in AB and UCB. Plots of the total Lin CD127+ population of two representative samples are depicted on the left, while MFI for the respective markers in Lin CD127-CD161+ or Lin CD127-CD161- cells is plotted on the right. Numbers in the plots represent the percentage of cells as part of the total CD127+Lin- population. *: p < 0.05, **: p < 0.01, Paired student’s T-test.
subset of ILC3s in humans\textsuperscript{23}. Both CD62L and CD161 are highly expressed on human adult ILC2s\textsuperscript{38}. All markers were found to be expressed on ILCs in both AB and UCB (Fig. 2A-D). A larger percentage of the ILC population in UCB expressed CD161, which was also reflected in the expression levels per cell, as judged by mean fluorescence intensity (MFI) (Fig. 2A). Although in both UCB and AB ILC2s expressed this marker uniformly as published previously\textsuperscript{13,38}, UCB ILC2s expressed higher levels of CD161 per cell (Fig. 2A). The higher expression of CD161 in total UCB ILCs may be a reflection of the higher percentage of ILC2s in UCB as compared to AB (Fig. 1B) as ILC1s and ILC3s express similar levels of CD161 in UCB and AB (Fig. 2A). While the role of CD161 in NK cells and T cells becomes exceedingly clear\textsuperscript{30-32}, its function in ILC biology is currently unknown. CD62L and CD45RA have been shown to mark naïve-like ILC3s in human tonsils\textsuperscript{23} and α4β7 is expressed by ILC precursors in mice\textsuperscript{25,26,29}. In both UCB and AB a similar portion of the ILC population expressed CD62L, although, strikingly, ILCs in AB expressed higher levels of CD62L per cell (Fig. 2B). As opposed to CD62L, more CD45RA expressing ILCs, and more specifically ILC1s were found in UCB, while ILC2s and ILC3s almost uniformly expressed this marker in both AB and UCB. However, per-cell expression of CD45RA was higher in UCB as compared to AB in all populations (Fig. 2C). No significant differences in percentages of integrin α4β7 expressing ILCs were found between UCB and AB ILCs with the exception of ILC2s, as more α4β7 expressing ILCs were present in UCB. Interestingly, like CD62L, α4β7 expression level was higher on AB as compared to UCB ILCs (Fig. 2D). It could generally be expected that UCB contains more naïve-like cells in comparison to effector-like cells. However, lower expression levels of CD62L and α4β7 seem to contradict this naïve-like state, while the expression patterns of CD45RA is in line with this. Importantly, CD62L and α4β7 are homing receptors to the MALT and SLOs, while CD45RA is not. Differential expression of CD62L and α4β7 can thus simply reflect altered homing of the cells, necessary for ILC seeding of the various tissues in perinatal life. In line with this and with the expectation that UCB contains cells that are more ‘naïve-like’ as compared to AB, the expression of CD45RA per cell as measured by mean fluorescence intensity (MFI) is higher in UCB as compared to AB (Fig. 2C). Furthermore, it is interesting in this respect that in both UCB and AB, a negative correlation was seen between expression of CD45RA and CD161 (Fig. 2E). Although all CD161\textsuperscript{+} cells expressed CD45RA, it was at lower levels as compared with CD161\textsuperscript{+} cells. As mentioned before, CD45RA is a marker for naïve like, immature, ILCs\textsuperscript{23} and CD161 marks cytokine producing, effector T cells\textsuperscript{31,32}. As UCB ILCs which can be expected to be enriched for less matured ILCs express higher levels of CD161, its role in ILC biology remains an issue for further investigation. Intriguingly, while in AB expression of α4β7 positively
correlates with expression levels of CD161 the exact opposite is true for UCB (Fig. 2E), further emphasizing that α4β7 might mark different populations of ILCs in UCB as compared to AB.

A population of CD62L⁺α4β7⁺ ILCs is enriched in UCB

As mentioned previously, α4β7 marks ILC precursors in both fetal and adult mice. Its ligand (MAdCAM) is expressed on the high endothelial venules (HEV) of all SLOs in both mouse and human neonates, while in adults it is only expressed on the HEVs of MALT. As such, in adults α4β7 marks cells migrating to the MALT and the intestine. Indeed, while expression of α4β7 on UCB ILCs positively correlates with CD62L, a homing molecule to both peripheral SLOs and MALT, such a correlation is not observed in AB (Fig. 3A). As expression of α4β7 is positively correlated with CD161 in AB (Fig. 2E), it is tempting to speculate that the cells migrating to the MALT in adults are mature effector ILCs, which were instructed to migrate to the MALT in either bone marrow or SLOs. In contrast, in UCB α4β7, which marks a population of immature ILC precursors in mice, presumably marks less mature, potential precursor, ILCs. This is supported by the positive correlation between expression of α4β7 and CD62L in UCB and the observation that a population expressing both markers was present in UCB while mostly absent in AB (Fig. 3B). We further reasoned that if indeed true that CD62L⁺α4β7⁺ ILCs can be viewed as precursors and considered a separate population, that the distribution of the three different subsets upon excluding this population would resemble AB more closely. Indeed, while all three ILC subsets were represented within the Lin⁻CD127⁺CD62L⁺ population, this population was enriched for CRTH2⁺ ILCs at the expense of ILC3s (Fig. 3C). When excluded, the relative enrichment of ILC2s in UCB was not observed any more (Fig. 3C). Furthermore, CD62L⁺α4β7⁺ ILC2s expressed higher levels of CRTH2 as compared to the other ILC2 subsets investigated (Fig. 3D). As expression of CRTH2 is directly regulated by GATA3, this shows that these cells express the highest levels of GATA3. GATA3 is expressed by all ILC subsets to a certain degree and is crucial for the bone marrow development of all ILCs in mice. As ILCs in circulation are less matured than their counterparts in epithelial tissues, it is questionable whether in this specific case GATA3 (and thus CRTH2) expression correlates with ILC2 effector functions or that it marks not yet terminally differentiated ILC precursors. These results lead to a model in which a α4β7⁺CD62L⁺ pre-ILC can mature into a circulating α4β7⁺CD62L⁺ ILC (ILC2). In parallel with naïve T cells, the latter would then be able to gain homing capacities to all tissues. In case of activation and subsequent homing to mucosal tissues, ILC2 would lose expression of CD62L while regaining α4β7, thus maturing into α4β7⁺CD62L⁻ mucosa homing ILCs (ILCm). Based on our results and
in line with previous observations, GATA3 expression would be highest in the pre-ILC and decline during the maturation steps. Only in case of final differentiation to ILC2 will GATA3 levels go back up.
Our results further highlight the different roles of α4β7 in neonates as compared to adult, with the presumed specific role for α4β7 to seed different tissues in which MAdCAM-1 is expressed with ILC precursor type cells. Further experiments will have to be carried out to determine whether the CD62L⁺α4β7⁺ population indeed forms a population of human ILC precursors. At the same time, whether expression of CD45RA and CD161 genuinely marks different maturation stages of ILCs in either adults or neonates will have to be addressed in the future using multi-parameter analysis, e.g. mass-cytometry and/or single-cell RNA sequencing.

In conclusion, we found that the composition of the ILC population in AB as compared to UCB is indeed different. As such it is interesting to hypothesize that a fully matured commensal flora influences the ILC population in human circulation. UCB is enriched for a population of CD62L⁺α4β7⁺ ILCs which could potentially be viewed as a human precursor ILC population, although this should be determined in future experiments. Importantly, our investigation was focused on circulating ILCs, which in adults are less functional than tissue resident ILCs⁴⁸ (this thesis, chapter 3). Future examination of ILCs from human fetal tissue in comparison with adult counterparts (e.g. intestines, skin) will have to further illuminate the effect of a fully matured commensal flora on human ILCs.

**Materials and Methods**

**Umbilical cord blood and adult peripheral blood**

UCB was obtained from the department of Obstetrics and Gynaecology, VU University Medical Center, Amsterdam, the Netherlands, upon receiving signed informed consent. Peripheral blood from healthy adults (AB) was obtained upon receiving signed informed consent. All blood samples were collected according to the guidelines of the Medical Ethical Committee of the VU University Medical Center (Amsterdam, The Netherlands), in accordance with the Declaration of Helsinki and according to Dutch law. AB and UCB mononuclear cells (MNCs) were isolated by gradient centrifugation on lymphoprep (d = 1.077, Fresenius Kabi Norge AS). The MNCs were subsequently centrifuged and washed twice in cold PBS (B. Braun Melsungen AG) supplemented with 10% (v/v) donor plasma.

**Flow cytometry**

For flow cytometry, cells were stained with the following antibodies: CD3 (UCHT1), CD11c (3.9), CD19 (HIB19), CD14 (61D3), CD34 (4H11), CD94 (DX22, all FITC labeled), CD127-PE (eBioRDR5), CD117-PeCy7 (104D2, all eBioscience, San Diego, CA, USA), CRTH2-PE-CF594 (BM16), CD161- BV510
(DX12), CD62L-BV421 (DREG56, all BD Bioscience, Mountain View, CA, USA) and CD45RA-APC (HI100, Biolegend, San Diego, CA, USA). The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: α4-β7 monoclonal antibody (Act-1, cat#11718) from Dr. A. A. Ansari. The antibody was biotin labeled in house using EZ-link™ NHS Biotin (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturers’ protocol. Biotin labeling was detected using streptavidin, AlexaFluor® 647 (Thermo Fisher Scientific, Waltham, MA, USA). Exclusion of dead cells was done using staining with Fixable Viability Dye eFluor® 780 (eBioscience, San Diego, CA, USA). Analysis was performed using either LSR-Fortessa X20 (BD Bioscience, Mountain View, CA, USA). Further analysis was done using Flowjo Software version 10. for Microsoft (Tree Star, San Carlos, CA). Gating was done based on Fluorescence Minus One (FMO) controls.

Statistics
Results are given as the mean ± SD. Statistical analysis was done using GraphPad Prism 5 Software (La Jolla, CA, USA). Significance is indicated by * p ≤ 0.05, ** p < 0.01, *** p<0.001 or **** p<0.0001. We did not use statistical methods to predetermine sample size of human samples, nor were the investigators blinded to sample identity or results. Samples were not randomized.
References

Comparison of UCB and AB ILCs


